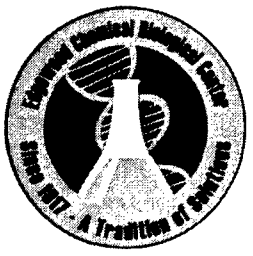

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ECBC-TR-485

TOXICITY OF A NEW POLYNITRAMINE ENERGETIC MATERIAL, CL-20, TO THE ENCHYTRAEID WORM, *ENCHYTRAEUS CRYPTICUS,* IN A SANDY LOAM SOIL

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The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorizing documents.

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In this report, the toxicity of polynitramine energetic material, hexanitrohexaazaisowurtzitane (CL-20) to the soil invertebrate species, *Enchytraeus crypticus,* was investigated using the Enchytraeid Reproduction Test (ISO/16387:2003). The study was designed to develop ecotoxicological benchmark values for the ecological risk assessment of the potential impacts of the accidental release of this compound into the environment. Tests were conducted in Sassafras Sandy Loam soil, which supports the relatively high bioavailability of CL-20. Weathering and aging procedures for CL-20 amended into test soil were incorporated into the study design to produce toxicity data that better reflect soil exposure conditions in the field. Results showed that toxicities for *E. crypticus* adult survival and juvenile production were significantly increased in weathered and aged treatments compared with toxicity in freshly amended soil, based on 95% confidence intervals. The EC_{50} and EC_{20} values for juvenile production were 0.3 and 0.1 mg kg^{-1}, respectively, for CL-20 freshly amended into soil, and 0.1 and 0.035 mg kg^{-1}, respectively, for weathered and aged CL-20 treatments. These findings of increased toxicity to *E. crypticus* in weathered and aged CL-20 treatments show that future investigations should include a weathering and aging component to generate toxicity data that provide more complete information on the ecotoxicological effects of emerging energetic contaminants in soil.

15. SUBJECT TERMS

Hexanitrohexaazaisowurtzitane	Toxicity assessment	Weathering/aging	CL-20
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PREFACE

The work described in this report was authorized under Project No. SERDP CP-1254. The work was started in July 2002 and completed in August 2005.

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CONTENTS

FIGURES

TABLES

TOXICITY OF A NEW POLYNITRAMINE ENERGETIC MATERIAL, CL-20, TO THE ENCHYTRAEID WORM, *ENCHYTRAEUS CRYPTICUS,* IN A SANDY LOAM SOIL

1. INTRODUCTION

Energetic materials (Ems) are employed in a wide range of commercial and military activities, and are often released into the environment, leading to the contamination of soil and groundwater (Simini et al., 1995; Jenkins et al., 2001). Such activities are also likely routes for environmental contamination by an emerging cyclic nitramine explosive and propellant material, 2,4,6,8,10,12-hexanitrohexaazoisowurtizane (HNIW or CL-20), which was synthesized by Nielsen et al. (1987, 1998) and is being considered as a potential replacement for existing cyclic nitramine explosives hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX). Little is known about the fate of CL-20 in soil, and understanding of potential ecological impacts of its accidental release in the environment is necessary prior to large-scale production of CL-20.

Similar to RDX and HMX, CL-20 contains multiple electron-withdrawing N-NO_2 functional groups (Figure 1), causing the explosive to resist electrophilic attack by oxygenases under aerobic soil conditions resulting in slow and incomplete mineralization (Hawari, 1999; Hawari et al., 2004). CL-20 was hypothesized to have ecotoxicological effects on soil organisms inhabiting vadose zone similar to those of RDX and HMX. Similarities in chemical structure-toxicity relationships were evident in studies conducted with cyclic nitramines RDX or HMX, both of which showed no adverse effect on adult earthworms *Eisenia fetida* or *Eisenia andrei* up to 500 mg kg^{-1} in the Organization for Economic Co-operation and Development (OECD) artificial soil (OECD, 1984), similarly formulated USEPA Standard Artificial Soil (U.S. Environmental Protection Agency; USEPA, 1989), and in natural soils (Phillips et al., 1993; Robidoux et al., 2002), with low toxicities to enchytraeid worm (potworm) *Enchytraeus crypticus* as indicated by EC_{20} value for juvenile production of 3,700 mg kg^{-1} RDX, and an unbounded no observable effect concentration (NOEC) for either adult survival or juvenile production of 2,1750 mg kg^{-1} HMX in a natural sandy loam soil (Kuperman et al., 2003). Similarly, NOEC values of 1,000 mg kg^{-1} RDX or HMX were reported by Schäfer and Achazi (1999) for either adult survival or reproduction of *E. crypticus* in standard LUFA 2.2 soil.

Hypothesized ecotoxicological similarity of CL-20 with that of RDX or HMX, was in agreement with limited published data that showed CL-20 did not adversely affect soil microbial activity (measured as dehydrogenase and potential nitrification activities), or terrestrial plants alfalfa (*Medicago sativa*) and perennial grass (*Lolium perenne*) as assessed by seed germination and early plant growth up to 10000 mg kg^{-1} (Gong et al., 2004). However in dramatic contrast to prediction, CL-20 was highly toxic to potworms *E. crypticus* and *E. albidus* with EC_{50} values for juvenile production in different soil types ranging from 0.08 to 0.62 mg kg^{-1} (Dodard et al., 2005), and earthworm *Eisenia andrei* with EC_{50} values for reproduction endpoints ranging from 0.05 to 0.09 mg kg^{-1} (Robidoux et al., 2004). Notwithstanding that toxicological data from both studies was based on nominal CL-20 concentrations and on exposures only in freshly amended soils, these contrasting ecotoxicological effects clearly demonstrated that

attempts to predict the potential ecotoxicity of CL-20 solely on the basis of similarities in structure and functional groups to those of RDX or HMX can lead to incorrect conclusions regarding ecological impacts of CL-20 in the environment. Recognizing a need for quantifying ecotoxicological benchmarks that can be used for development of scientifically based Ecological Soil Screening Levels (Eco-SSLs; USEPA, 2005), we conducted this research as part of a larger project aimed at advancing the knowledge of the CL-20 toxicity to ecological receptors.

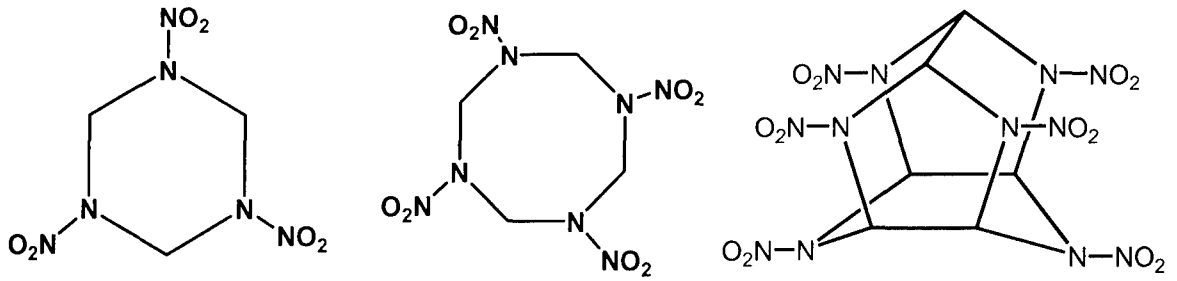

Figure 1. Chemical Structures of the Three Cyclic Nitramines, RDX, HMX, and CL-20.

Development of toxicity benchmarks that adequately reflect potential ecological risks requires assessment of the effects of weathering and aging of contaminant explosives in soil on the exposed soil organisms. Weathering and aging of explosives in soil may reduce exposure of soil invertebrates due to photodecomposition, hydrolysis, reaction with organic matter, sorption/fixation, precipitation, immobilization, occlusion, microbial transformation, and other fate processes that commonly occur at contaminated sites (Kaplan, 1992; Gorontzy et al., 1994; Preuß and Rieger, 1995; Daun et al., 1998; Hawari et al., 1998; Alexander, 2000; Spain et al., 2000; Dodard et al., 2004; Kuperman et al., 2005). Certain fate processes, including microbial transformation of explosives, can also produce chemicals that are more bioavailable or more toxic to soil organisms compared with parent compounds freshly introduced into soil. In order to investigate the net ecotoxicological effects on soil biota including results of possible chemical alteration in the soil environment, we designed our studies to test the hypothesis that weathering and aging of CL-20 in natural soil can alter the exposure effects on test organisms compared with effects in freshly amended soil. This was accomplished by conducting definitive toxicity assays on soil containing CL-20 freshly amended into the soil, and for CL-20 weathered and aged in soil, then statistically comparing toxicological data determined in both exposure types.

2. MATERIALS AND METHODS

This study was designed to meet specific criteria for toxicity benchmark data required for the development of Eco-SSLs (USEPA, 2005), including: (1) tests were conducted in soil having physico-chemical characteristics that support relatively high bioavailability of chemicals; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species were specified and appropriate. Compliance with these criteria insures sufficient quality of toxicity benchmark data for Eco-SSL development and has been used in several investigations of EM toxicity for both soil invertebrates (Kuperman, et al., 2003, 2005; Simini et al., 2003) and terrestrial plants (Rocheleau et al., 2005).

2.1 Test Soil.

A natural soil, Sassafras Sandy Loam [Fine-loamy, siliceous, mesic Typic Hapludult] (USDA/ARS, 1999; SSL) was used in this study to assess CL-20 toxicity to *E. crypticus*. This soil was selected for developing ecotoxicological values protective of soil biota because it has physical and chemical characteristics supporting relatively high bioavailability of explosives (USEPA, 2005), including low organic matter and clay contents (69% sand, 13% silt, 17% clay, 1.2% organic matter, 5.5 cmol kg^{-1} cation-exchange capacity, pH 5.2). Total concentrations of metals and nutrients were within regional background ranges, and were reported previously (Robidoux et al., 2004). The SSL soil was collected from an open grassland field on the property of the U.S. Army Aberdeen Proving Ground (APG; Edgewood, MD, USA). The soil was sieved through a 5-mm mesh screen, air-dried for at least 72 hours and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then stored at room temperature before use in testing. Detailed procedures are described in Kuperman et al. (2003, 2004, 2005).

2.2 Chemicals and Reagents.

Crystalline CL-20 (CAS 135285-90-4; ε-isomer, purity 99.3%) was obtained from ATK Thiokol Propulsion (Ogden, UT, USA). Beryllium sulfate ($BeSO_4 \cdot 4H_2O$, CAS 7787-56-6, purity 99.99%, Alfa Aesar, Ward Hill, MA, USA) was used as the positive control in these tests. Acetone (CAS 67-64-1, HPLC Grade, Fisher Scientific, Pittsburgh, PA, USA) was used for preparing CL-20 solutions during soil amendments. Acetonitrile (CAS 75-05-8, HPLC Grade, Pharmco, Brookfield, CT, USA) was used for chemical extractions and for subsequent analyses. Methanol (CAS 67-56-1, Chromatography grade, purity 99.9%, Pharmco, Brookfield, CT, USA) was used in determinations by HPLC. Ethanol (CAS: 64-17-5, purity 99.98%; Pharmco, Brookfield, CT, USA) was used as preservative for potworms. Calcium chloride ($CaCl_2 \cdot 2H_2O$, CAS: 10043-52-4, reagent grade 100%, J.T. Baker, Phillipsburg, NJ, USA). Sodium bisulfate monohydrate ($NaHSO_4 \cdot H_2O$, CAS: 10034-88-5, purity 99%; SIGMA-ALDRICH, St Louis, MO, USA). Rose Bengal biological stain (CAS: 632-68-8, dye content 80%, Fisher Scientific,

Pittsburgh, PA, USA) was used for staining potworm tissues. Unless otherwise specified, American Society of Testing and Materials (ASTM) Type I water (ASTM, 2004) obtained using Milli-RO® 10 Plus followed by Milli-Q® PF Plus systems (Millipore®, Bedford, MA, USA) was used throughout the studies. Glassware was washed with phosphate-free detergent, followed by rinses with tap water, ASTM Type II water, analytical reagent grade nitric acid 1% (volume/volume), then with ASTM Type I water.

2.3 Soil Amendment Procedures.

Studies were performed separately and independently for CL-20 in freshly amended soil, and for amended CL-20 that was weathered and aged in soil, in order to determine toxicity benchmark values for CL-20-contaminated soil of each exposure type. Individual CL-20 treatment levels in SSL soil were prepared as single batches for toxicity studies. Each soil batch representing a specific CL-20 treatment concentration was the source of the exposure substrate for definitive tests of CL-20 freshly amended to SSL, substrate for weathering and aging CL-20 in SSL, and each was analyzed to determine CL-20 concentration at the time of introducing test species. During treatment batch preparation, CL-20 was mixed into soil using an organic solvent as a carrier, necessary to dissolve the nonpolar chemical in order to yield a more homogeneous mixture than direct addition of solid chemical crystals to soil. CL-20 was dissolved into acetone and pipetted onto a 2.5 cm thick layer of soil to establish an initial soil concentrate. The acetone was allowed to volatilize for minimum of 18 h. Photolysis of CL-20 was controlled by volatilizing the acetone in a dark chemical hood. Amended soil was transferred into a fluorocarbon-coated high-density polyethylene container and mixed for 18 hours on a three-dimensional rotary soil mixer. The final treatment batches were prepared by mixing initially-prepared soil concentrate of CL-20 with clean SSL soil in test-required proportions for 18 hours on a three-dimensional rotary mixer. Carrier control soils were treated with acetone only. After mixing, soil was hydrated with ASTM Type I water to 100% of the soil water holding capacity (WHC; 18% water, on the basis of the dry SSL soil mass, DM) for toxicity testing of CL-20 freshly amended to SSL, or 60% of the WHC for the weathering and aging procedure. Hydrated soils prepared for toxicity tests were allowed to equilibrate for 24 h before exposing potworms.

2.4 Treatment Concentrations.

A range-finding test was conducted with freshly amended soil to determine treatment concentrations for a definitive test. Nominal CL-20 concentrations selected for the range-finding test were 0, 1, 5, 10, 50, 100, and 500 mg kg^{-1}. Data from the range finding test were used to determine the treatment concentrations for the definitive tests, which were conducted in freshly amended and weathered/aged amended SSL soil using nominal CL-20 concentrations: 0, 0.1, 0.25, 0.5, 0.75, 1, 5, and 10 mg kg^{-1}. An additional nominal concentration of 0.08 mg kg^{-1} CL-20 was used in a test with freshly amended SSL soil. Positive controls in the form of a solution of beryllium sulfate were prepared in ASTM Type I water using 45 mg kg^{-1} Be, a nominal concentration, in all the tests. Nominal CL-20 test concentrations were analytically verified using modified USEPA Method 8330 (USEPA, 1998).

2.5 Weathering and Aging of Amended Soil.

Weathering and aging of CL-20 in soil was conducted according to procedures described by Kuperman et al. (2003, 2004, 2005). These procedures included exposing treated and control soil batches, initially hydrated to 60 percent of the WHC, in open glass containers in the greenhouse at ambient temperature to alternating wetting and drying cycles for twenty weeks. Concentrations of CL-20 in treatment soil batches with nominal concentrations 0.5, 1, and
10 mg kg^{-1} were analytically determined after 1, 6, 8, 15, 17, and 20 weeks of weathering and aging process to determine when the rate of change in CL-20 concentrations substantially declined. This time of duration was then designated for terminating weathering and aging procedures, and for commencement of definitive toxicity testing. During the weathering and aging procedure all soil treatments were weighed and readjusted to their initial mass at 60% of the WHC by adding ASTM Type I water each week to the soil. All soil treatments were brought to 100% of the WHC 24 h prior to commencement of potworm toxicity tests.

2.6 Chemical Extractions and Analyses.

Soil samples for analysis were taken after the 24-h moisture equilibration, at the beginning of each definitive test. From each treatment soil batch, 2.3 g of hydrated soil was weighed in triplicate into a 16-ml glass tube, 10 ml acetonitrile was added and the samples were vortexed for 1 min, then sonicated in the dark for 18 hours at 20°C. Sonicated samples were centrifuged at 2700 rpm for 30 minutes. Five mL of supernatant were transferred to a 20-ml glass vial and combined with 5 ml of $CaCl_2/NaHSO_4$ aqueous solution (5 and 0.2 g l^{-1}, respectively). The samples were shaken and left to equilibrate and settle for 30 minutes. The supernatant was filtered using disposable syringes (10 ml) and 0.45-μm Millipore polytetrafluoroethylene (PTFE) syringe filters. The first 3 ml of filtrate was discarded, and the remainder was retained in a PTFE-capped 4-ml vial. One mL of this filtered solution was transferred to a HPLC vial. The filtered samples were stored in the refrigerator at 4°C in the dark no longer than five days if not analyzed on the same day. Soil extracts were analyzed and quantified using a modified EPA Method 8330A (USEPA, 1998). The results of acetonitrile soil extractions are reported as CL-20 concentrations in dry soil.

Concentrations of CL-20 in the soil extracts were determined using the HPLC-UV system, which consisted of an Agilent 1100 HPLC Series equipped with a Supelcosil LC-CN column (25 cm x 4.6 mm x 5 μm), employing an isocratic 70:30 methanol: water mobile phase with a flow rate of 1.0 ml min^{-1} and a 50 μl injection volume. The autosampler was set at 10°C. Blanks and standards were placed among samples having unknown concentration in order to maintain quality assurance of the samples. Detection of CL-20 was accomplished using a diode array detector at 230 nm wavelength. A primary stock solution was prepared at 10,000 mg l^{-1} CL-20 in acetonitrile. Intermediate stock solutions of 50, 20, 2, 0.5, and 0.1 mg l^{-1} CL-20 in acetonitrile were then prepared from the primary stock solution. Calibration standards were made from the intermediate stock solutions with acidified water (sodium bisulfate) solution (50:50) to yield standards of 25, 10, 1, 0.25, and 0.05 mg l^{-1} CL-20 in acetonitrile /H_3O^+. Calibration curves were created (r^2>0.99999) with an instrument limit of detection (LOD) of 0.01 mg l^{-1} (S/N=3). Over five months, the reproducibility of the slope was determined to be

149.0 ± 5.0 with a %RSD of 3.4 (n=14). The lowest concentration of CL-20 that could be detected in freshly amended SSL soil was in the nominal treatment of 0.08 mg kg^{-1}, and was 0.098 mg kg^{-1}. The lowest detectable concentration of CL-20 weathered and aged in SSL soil was in the nominal treatment of 0.1 mg kg^{-1}, corresponding to 0.06 mg kg^{-1}.

2.7 Toxicity Assessment.

The primary objective of this study was to quantify CL-20 toxicity to the soil invertebrate *E. crypticus* for production of toxicity benchmark data that can be used in the development of Eco-SSL for soil invertebrates. Definitive tests were conducted with either freshly amended or with CL-20 weathered and aged in SSL soil. Nominal concentrations [mg kg^{-1}] ranged from 0.08 to 10 in tests with freshly amended soil, and from 0.1 to 10 in tests with CL-20 weathered and aged in soil (Table 1). All definitive tests included negative control (no chemicals added), carrier (acetone) control, and positive control. Positive control was prepared as solution of beryllium sulfate in ASTM Type I water using 45 mg kg^{-1} Be nominal concentration.

The Enchytraeid Reproduction Test was used to assess the effects of CL-20 on the adult survival and reproduction of the enchytraeid worm, *E. crypticus*. The test is an adaptation of an International Standardization Organization (ISO) bioassay ISO/16387 (ISO, 2003), which was modified for use with natural soils as described in Kuperman et al. (2003, 2004, 2005). The modifications were as follows:

(i) different soil hydration levels (described above) required for SSL soil, that has lower WHC compared with Standard Artificial soil, and

(ii) shorter test duration for *E. crypticus* (28 d vs. 42 d) due to shorter generation time of this species, compared with *E. albidus* for which ISO/16387 test conditions were originally optimized.

Tests were conducted in glass jars (42 mm ID; 45 mm deep) containing twenty grams of prepared soil, to which 0.05 g of ground oats were added as food for potworms. Each treatment and controls were replicated four times. Adult potworms with eggs in the clitellum region were collected from culture maintained in the same SSL soil type. Ten enchytraeid worms selected for uniformity (approximately 1 cm in length) were placed on top of the prepared soil in each test container. Plastic wrap was stretched over the top of each container and was perforated with three pinholes to facilitate air exchange. All containers were randomly placed in an environment-controlled incubator at 22 ± 1°C and 16:8 h light/dark photoperiod. The containers were weighed once a week and the mass loss was replenished with the appropriate amount of ASTM Type I water. Ground oats (0.05 g) were again added to each test container at that time.

Soil in each jar was carefully searched after 14 d to remove and count adult potworms. The remaining test substrate, including any cocoons laid during the first 14 d of the test, was incubated for an additional 14 d. After 28 d from the start of the test, soil in the test jars was fixed with 70% ethanol, and nine drops of Rose Bengal biological stain (1% solution in

ethanol) were added. Staining continued for minimum of 24 h. The content of each test jar was wet-sieved using a No. 100 mesh (150 μm) sieve, and retained contents transferred to a counting tray where potworms were counted. Measurement endpoints included number of surviving adults after 14 d, and number of juveniles produced after 28 d. All ecotoxicological parameters were determined using measured CL-20 concentration for each treatment level.

Validity criteria were included in the test as part of the Quality Control procedures. They included the following performance parameters for the negative controls: the adult mortality does not exceed 20% after 14 d; the average number of juvenile potworms per test container at the end of the test is greater than 2.5-times the initial number of adult potworms per test container; and the coefficient of variation for the mean number of juveniles is ≤ 50% at the end of the test. Test results complied with these validity criteria, defined in the ISO 16387 guideline. Mean adult survival in negative controls was 100% in both definitive tests. The mean numbers of juveniles in negative controls of freshly amended, and weathered and aged treatments, respectively, were 1685 and 1973, and the coefficients of variation were 8 and 15 percent. Juvenile production in positive controls was reduced by 56 and 59 percent from respective negative controls, and was within ± 2 times standard error of the positive control baseline established for the laboratory culture of *E. crypticus*. These results confirmed the power of the test, indicating that the toxicological effects determined in the definitive tests were most likely due to CL-20 treatments.

2.8 Data Analysis.

Adult survival and juvenile production data were analyzed using nonlinear regression models described in Stephenson et al. (2000). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether to weight the data, and to select potential models. The logistic (Gompertz) model (1) had the best fit for adult survival data in toxicity test with freshly amended SSL soil. Exponential model (2) had the best fit for the juvenile production data in both exposure types. The best fit of the lines generated by these models were closest to the data points, the variances of residuals were the smallest, and the residuals had the best appearance (i.e., most random scattering). These models were:

$$Y = a \times e([\log(1\text{-}p)] \times [C/\mathrm{ECp}]^b) \quad (1)$$

$$Y = a \times e(([\log(1\text{-}p)] / \mathrm{ECp}) \times C) + b \quad (2)$$

where Y = number for a measurement endpoint (e.g., number of juveniles), a = control response, e = base of the natural logarithm, p = inhibition/100 (e.g., 0.50 for EC_{50}), C = exposure concentration in test soil, ECp = estimate of effect concentration for a specified percent effect, and b = scale parameter. The ECp parameters used in this study included CL-20 concentration producing a 20% (EC_{20}) or 50% (EC_{50}) reduction in the measurement endpoint. The EC_{20} parameter based on a reproduction endpoint is the preferred parameter for deriving soil invertebrate Eco-SSL values (USEPA, 2005). The EC_{50}, a commonly reported value, and survival data were included to enable comparisons of the results produced in this study with results reported by other researchers. The 95% confidence intervals (CI) associated with the

point estimates were determined. Values for regression coefficients (R^2) determined for all ECp endpoints were greater than 0.948 indicating good fit of the model used for adult survival (logistic) or juvenile production (exponential) data.

Analysis of Variance (ANOVA) was used to determine the bounded (when possible) NOEC and Lowest Observed Effect Concentration (LOEC) values for adult survival or juvenile production data. Mean separations were done using Fisher's Least Significant Difference (LSD) pairwise comparison tests. A significance level of $p \leq 0.05$ was accepted for determining the NOEC and LOEC values. All analyses were done using measured CL-20 concentrations. Statistical analyses were performed using SYSTAT 7.0.1 (SPSS, 1997).

3. RESULTS AND DISCUSSION

3.1 Analytical Determinations of CL-20 in Soil.

Concentrations of CL-20 decreased in monitored 0.5, 1, and 10 mg kg^{-1} treatments during a 20-week weathering and aging procedure (Figure 2). The average (± standard error) recoveries of CL-20 among the three monitored treatments were 97±3, 88±3, 75±2, 76±3, and 70±3 percent of the initial concentrations after 6, 8, 15, 17, and 20 weeks of weathering and aging, respectively. The CL-20 recovery after 20 weeks was 68±8, 68±2, and 74±3 percent in 0.5, 1, and 10 mg kg^{-1} nominal treatments, respectively. Analytical determinations 20 weeks after initiation of the weathering and aging procedure showed that the rate of change in CL-20 concentrations substantially declined in treatments representing low, intermediate, and high levels of the exposure range selected for definitive testing. The definitive testing for CL-20 weathered and aged in SSL was initiated after total of 23 weeks from the start of the procedure with soils having approximately 60 percent of initial concentrations.

Analytically determined CL-20 concentrations in freshly amended soils used in definitive toxicity test averaged 108 percent (range from 97 to 123%) of nominal concentrations (Table 1), indicating good correlation between the nominal and measured CL-20 concentrations determined in our study after a 24-h equilibration period for soils hydrated to 100% of the WHC. This percent recovery was comparable with an average of 87% recovery determined by Robidoux et al. (2004) in their study with similar SSL soil immediately extracted following the amendment with nominal CL-20 concentrations ranging from 0.32 to 10 mg kg^{-1}. Overall, our results of chemical analyses confirmed that the soil amendment procedure used in toxicity tests was appropriate, and that the USEPA Method 8330A was efficient for quantifying the amount of CL-20 in soil.

Special consideration in assessing CL-20 toxicity was given to the effects of weathering and aging of CL-20 in soil on the exposure of soil receptors. Assessment of the CL-20 toxicity included studies with CL-20 weathered and aged in amended soils to more closely simulate the exposure effects in the field locations, where CL-20 may persist for extended periods of time. CL-20 concentrations in soil decreased 43%, on average, in response to weathering and aging, with individual recovery percentages ranging from 43 to 67%, compared with initial concentrations in freshly amended soils (Table 1). Primary efforts of this

phase of investigation were focused on establishing the net toxic effects on *E. crypticus* from exposure to contaminated soil that may be attributed to the presence of CL-20 in an aerobic soil environment; hence identification of breakdown products of CL-20 weathered and aged in soils was not included in analytical determinations. Overall, chemical analyses demonstrated that chemical exposures for *E. crypticus* in soils amended with CL-20 and subjected to weathering and aging differed from those of soils containing freshly amended CL-20. The differing exposure conditions included decreased concentrations of CL-20, potential presence of any degradation products of CL-20 that could be formed during weathering and aging (Balakrishnan et al., 2003, 2004a, b; Trott et al., 2003), and potential alteration of CL-20 bioavailability in soil for the test species. The inclusion of the weathering and aging component in the toxicity assessments allowed us to incorporate potential alterations in the soil chemical environment, and corresponding changes in toxicity at contaminated sites, into the development of toxicological benchmarks for *E. crypticus*.

Table 1. Recovery of CL-20 from Sassafras Sandy Loam Soil in Freshly Amended or Weathered and Aged Treatments Used in Definitive Toxicity Tests.

Freshly amended treatments			Weathered and aged treatments	
Nominal	Determined Initial	Recovery	Determined Final	Final/Initial
mg kg^{-1}	mg kg^{-1}	%	mg kg^{-1}	%
0	BLOD[a]		BLOD	
0.08	0.098 (0.027)	123	NU[b]	
0.1	0.112 (0.003)	112	0.061 (0.002)	53
0.25	0.271 (0.004)	108	0.12 (0.03)	43
0.5	0.487 (0.031)	97	0.28 (0.01)	58
0.75	0.901 (0.033)	120	0.48 (0.01)	54
1	1.005 (0.008)	100	0.61 (0.02)	61
5	4.99 (0.09)	100	3.10 (0.01)	62
10	10.1 (0.4)	101	6.76 (0.14)	67

Table notes: Concentrations are based on acetonitrile extraction and HPLC using USEPA Method 8330A. Values are means (n = 3) and standard errors in parentheses. [a] BLOD = below limit of detection (LOD). LOD was 0.098 mg kg^{-1} dry soil in freshly amended treatments, and 0.06 mg kg^{-1} in weathered and aged treatments. [b] NU = treatment was not used in the toxicity study.

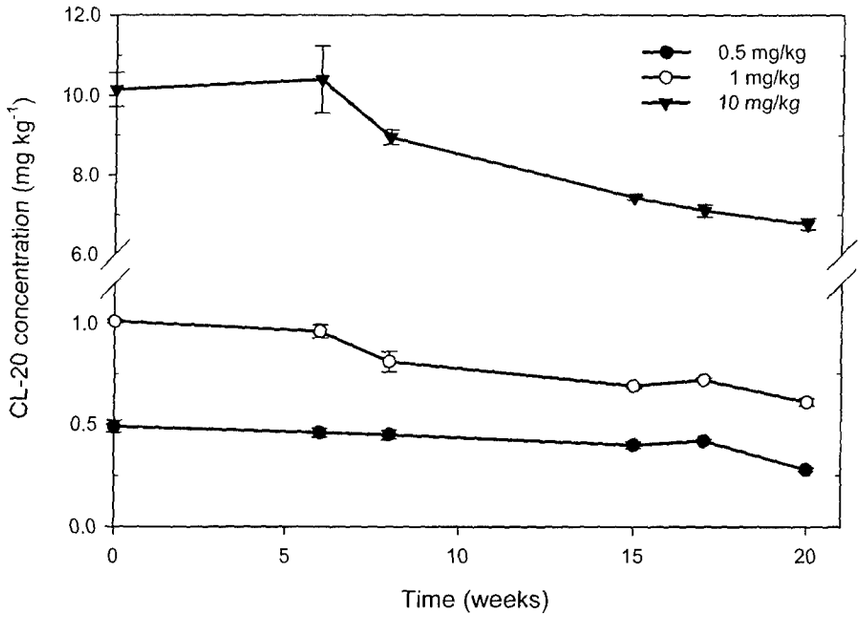

Figure 2. Changes in CL-20 Concentrations during Weathering and Aging in Sassafras Sandy Loam Soil Amended with 0.5, 1, and 10 mg kg^{-1} Nominal Treatments.

3.2 Range-Finding Toxicity Tests.

Measurement endpoints were assessed in the range-finding test using six treatment concentrations and two replicates per treatment. Potworms were exposed to nominal CL-20 concentrations of 0, 1, 5, 10, 50, 100, and 500 mg kg^{-1} using freshly amended SSL soil. Additional range-finding test was conducted with CL-20 weathered and aged in soil for one month using nominal CL-20 concentrations 0, 0.1, 0.5, 1.0, 5.0, and 10.0 mg kg^{-1} to determine concentration range for definitive test assessing the effects of weathering and aging of CL-20 in soil on toxicity. Adult *E. crypticus* survival in freshly amended SSL soil was initially significantly ($p = 0.049$) decreased in the 1 mg kg^{-1} treatment but was not adversely affected ($p > 0.05$) in the 5 or 10 mg kg^{-1} treatments compared with control. The decrease was 85 percent ($p < 0.0001$, LOEC) in the 50 mg kg^{-1} treatment. Juvenile production significantly ($p < 0.0001$) decreased in the first treatment concentration used producing an unbounded LOEC value of 1 mg kg^{-1}. The exponential model had the best fit for data. Juvenile production EC_{20} and EC_{50} values in freshly amended soil were 0.16 and 0.49 mg kg^{-1}, respectively.

Adult *E. crypticus* survival in test with CL-20 weathered and aged in SSL soil was significantly ($p < 0.01$) reduced in the 0.1 and 0.5 mg kg^{-1} treatments. Adult survival was not significantly ($p = 0.134$) different from control in the 1 mg kg^{-1} treatment and decreased significantly ($p \leq 0.041$) again in the 5 and 10 mg kg^{-1} treatments. Juvenile production in treatments with CL-20 weathered and aged in amended SSL soil followed a similar pattern. After a sharp decrease in the number of juveniles in the 0.1 and 0.5 mg kg^{-1} treatments, juvenile production increased in the 1 mg kg^{-1} treatment and decreased again in the 5 and 10 mg kg^{-1}

treatments. Decrease in juvenile numbers was significant ($p < 0.0001$) in all treatments compared with control, producing an unbounded LOEC value of 0.1 mg kg^{-1}. Juvenile production EC_{20} and EC_{50} values based on nominal concentrations were 0.009 and 0.027 mg kg^{-1}, respectively. These results suggested that weathering and aging of CL-20 in soil could increase the toxicity of CL-20 to *E. crypticus* when compared with the toxicity in freshly amended soil. The results of the range-finding tests were used to determine concentrations for definitive tests with *E. crypticus* in freshly amended as well as in weathered and aged CL-20 treatments.

3.3 Definitive Toxicity Tests.

Definitive studies were conducted to assess the effects of CL-20 on the enchytraeid worm *E. crypticus*. Adult potworms were exposed in independent investigations to a range of CL-20 concentrations in freshly amended soil, and CL-20 weathered and aged in soil. Adult *E. crypticus* survival and juvenile production were affected in freshly amended SSL within the concentrations range tested (Table 2). For adult survival, the bounded NOEC and LOEC values were 5 and 10 mg kg^{-1}, respectively. The EC_{20} and EC_{50} values for adult survival determined by logistic (Gompertz) model (Figure 3) were 6 and 18 mg kg^{-1}, respectively. Juvenile production was the more sensitive measurement endpoint for assessing CL-20 toxicity for *E. crypticus* compared with adult survival. The bounded NOEC and LOEC values for juvenile production were 0.11 and 0.27 mg kg^{-1}, respectively (Table 2). Concentration-response relationship for juvenile production determined by exponential model is shown in Figure 4. The EC_{20} and EC_{50} values for juvenile production were 0.1 and 0.3 mg kg^{-1}, respectively (Table 2).

Table 2. Summary of Toxicological Benchmarks (mg kg^{-1} Dry Soil) Determined for CL-20 Freshly Amended, and for CL-20 Weathered and Aged for 23 weeks, in Sassafras Sandy Loam Soil Using the Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

CL-20 exposure type	Adult survival				Juvenile production			
	NOEC	LOEC	EC_{20}	EC_{50}	NOEC	LOEC	EC_{20}	EC_{50}
Freshly amended	5	10	6	18	0.11	0.27	0.1	0.3
p or 95% CI	0.068	0.003	2-10	3-34	0.209	<0.0001	0.06-0.13	0.2-0.4
Weathered and aged	3.1	6.8	>6.8	>6.8	<0.06	0.06[b]	0.035	0.1
p or 95% CI	0.055	0.006	ND[a]	ND	ND	<0.0001	0.025-0.045	0.08-0.14

Table notes: Concentrations are based on acetonitrile extraction and HPLC using USEPA Method 8330A. Limit of detection was 0.098 mg kg^{-1} dry soil in freshly amended treatments, and 0.06 mg kg^{-1} in weathered and aged treatments. NOEC = no observed effect concentration; EC = effect concentration; CI = confidence intervals; [a] ND = parameter could not be determined within CL-20 concentration range tested; [b] Unbounded lowest observed effect concentration (LOEC) value.

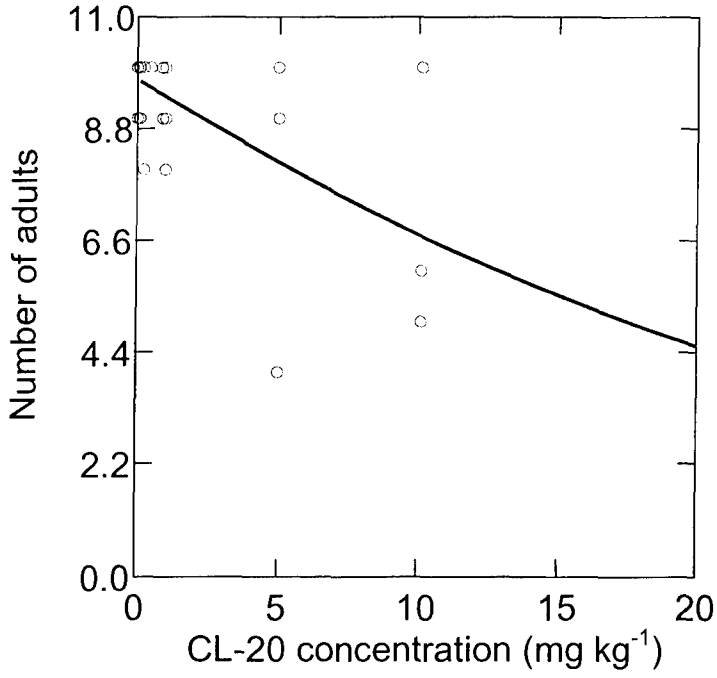

Figure 3. Effect of CL-20 on Adult *Enchytraeus crypticus* Survival in Freshly Amended Sassafras Sandy Loam Soil.

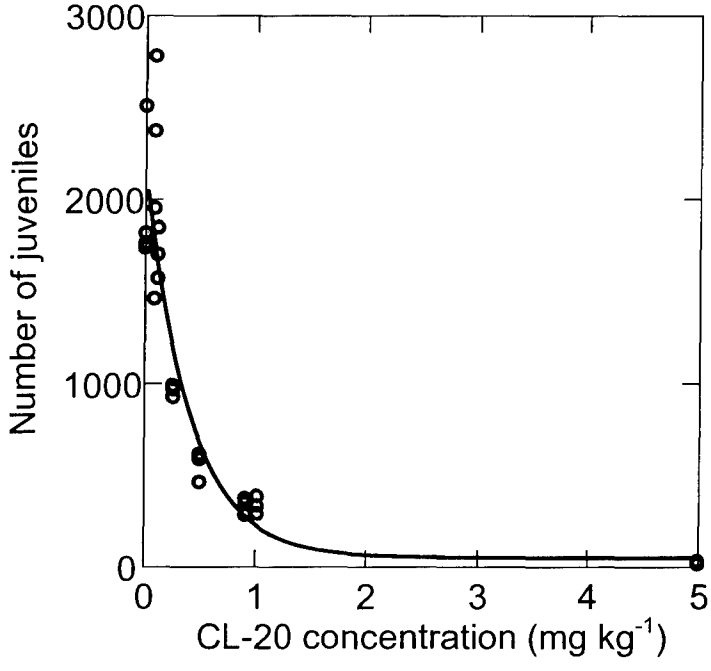

Figure 4. Effect of CL-20 on Juvenile Production by *Enchytraeus crypticus* in Freshly Amended Sassafras Sandy Loam Soil.

The bounded NOEC and LOEC values for *E. crypticus* adults exposed to CL-20 weathered and aged in SSL were 3.1 and 6.8 mg kg^{-1}, respectively (Table 2). Weathering and aging of CL-20 in SSL increased the toxicity of test soil for *E. crypticus* adults by 147 percent based on the bounded LOEC values. Concentration-response relationship for adult survival could not be determined for this treatment because adult survival decreased by only 15 percent compared with carrier control at the highest CL-20 concentration tested. Similar to the results of exposure in freshly amended soil, juvenile production in soil containing CL-20 weathered and aged in situ was more sensitive indicator of toxicity for *E. crypticus* compared with adult survival. This comports with results reported in literature for potworms (Schäfer and Achazi, 1999; Dodard et al., 2003; Kuperman et al., 2003, 2004, 2005; Römbke, 2003). A statistically significant ($p < 0.0001$) reduction in number of juveniles, compared with carrier control, occurred in the lowest analytically verifiable treatment producing an unbounded LOEC value of 0.06 mg kg^{-1} (Table 2). Increase in toxicity of weathered and aged CL-20 soil treatments was greater for the reproduction compared with adult survival based on LOEC values (Table 2). The exponential model had the best fit for juvenile production data (Figure 5), generating EC_{20} and EC_{50} values of 0.035 and 0.1 mg kg^{-1}, respectively (Table 2). These values were significantly (95% CI basis) lower compared with CL-20 effects in freshly amended soil, indicating an approximately 300 percent increase in toxicity of weathered and aged CL-20 treatments for reproduction of *E. crypticus*.

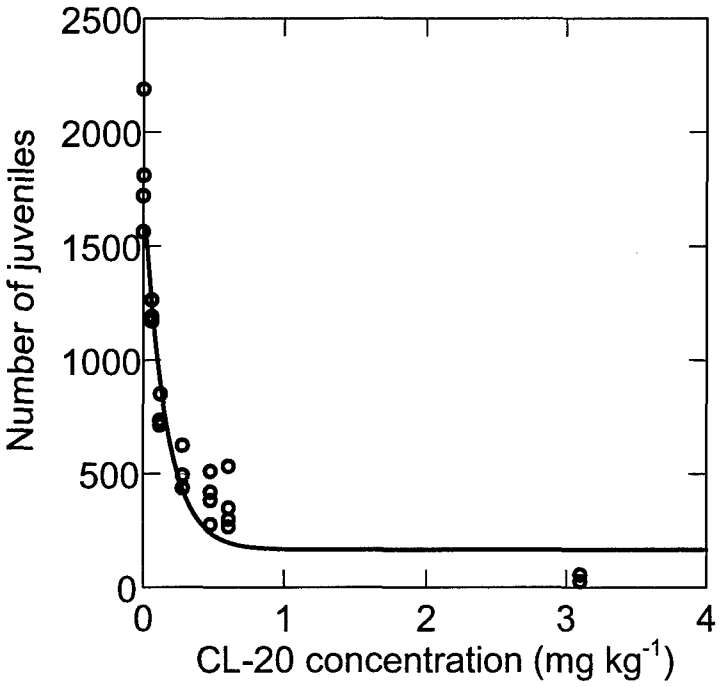

Figure 5. Effect of CL-20 Weathered and Aged in Sassafras Sandy Loam Soil on Juvenile Production by *Enchytraeus crypticus*.

Data for CL-20 toxicity to *E. crypticus* determined in the present study comport generally with data from the available published studies of CL-20 effects on enchytraeids (Dodard et al., 2005) and the earthworm *Eisenia andrei* (Robidoux et al., 2004). Dodard et al. (2005) reported lower toxicity benchmarks for *E. crypticus* in freshly amended SSL soil, including EC_{20} and EC_{50} values for juvenile production of 0.04 and 0.12 mg kg^{-1}, respectively based on nominal CL-20 concentrations. These values in two soil formulations with considerably greater pH and organic matter content ranged from 0.001 to 0.62 mg kg^{-1} nominal CL-20 (Dodard et al., 2005). Reproduction toxicity benchmarks for *E. crypticus* determined in our study with freshly amended SSL soil were within the 95% CI range of those determined by Dodard et al. (2005) for a different enchytraeid species, *E. albidus* in Rac50-50 soil formulation having 23% OM and pH 7.9, based on nominal CL-20 concentrations. Toxicity of CL-20 for adult *E. crypticus* was greater compared with toxicity to the earthworm *Eisenia andrei* based on NOEC, LOEC, LC_{20}, and LC_{50} values of 8.57, 90.7, 25.3, and 53.4 mg kg^{-1}, respectively determined in similar SSL soil freshly amended with CL-20 (Robidoux et al., 2004). Reproduction endpoints for *Eisenia andrei* (cocoon production and viability, juvenile production) were more sensitive to CL-20 exposure in freshly amended SSL based on EC_{50} values that ranged from 0.05 to 0.09 mg kg^{-1} (Robidoux et al., 2004) compared with our results for *E. crypticus*. However, these EC_{50} values were not statistically different (95% CI basis) from EC_{50} values for juvenile production by *E. crypticus* in weathered and aged CL-20 treatments determined in our study. Soil type affected the toxicity of CL-20 to *E. crypticus* (Dodard et al., 2005) and to *Eisenia andrei* (Robidoux et al., 2004). The reproduction toxicity benchmarks for *E. crypticus* were an order of magnitude lower (greater toxicity) in RacAg2002 soil formulation (41% OM, pH 8.2) compared with toxicity in SSL (Dodard et al., 2005). In contrast, all toxicological benchmarks for CL-20 were more than one order of magnitude greater (lower toxicity) for *Eisenia andrei* in similarly formulated RacFor2002 soil used in the study by Robidoux et al. (2004) compared with toxicity in SSL soil. The differential toxicity of CL-20 in these soil types suggests that future studies should include multiple soil types representing a broad range of soil properties (e.g., organic matter, clay content, pH) in order to assess the relationships among CL-20 toxicity, bioavailability, and soil properties.

Toxicological benchmark data determined in this study for *E. crypticus* shows that the toxicity of CL-20 was approximately two orders of magnitude greater compared with nitroaromatic energetic materials TNT, 2,4-DNT, 2,6-DNT, TNB, and more then five orders of magnitude greater compared with nitramine explosive RDX (Table 3), based on EC_{50} values for reproduction in the similarly designed studies with SSL soil (Kuperman et al., 2003, 2005). The difference in toxicity to *E. crypticus* between CL-20 and HMX was even greater (Table 3), where *E. crypticus* was not affected by exposure to HMX up to the highest tested concentration of 21750 mg kg^{-1} HMX in SSL soil (Kuperman et al., 2003). Based on the results of the present studies and those reported by others (Kuperman et al., 2003, 2005), the order of toxicity of cyclic nitramines to *E. crypticus* in SSL soil is (from greatest to least) CL-20 > RDX > HMX. This order of toxicity of the three explosives parallels closely the order of their respective log K_{ow} values (1.92 > 0.90 > 0.17; Monteil-Rivera et al., 2004) suggesting that greater toxicity of CL-20 can be related, at least partially, to its greater hydrophobicity and affinity toward organic matter, which increases its potential to partition into soil biota, and greater bioavailability and uptake potentials compared with either RDX or HMX.

Table 3. Reproduction Toxicity Benchmarks (mg kg^{-1}) for Explosive Soil Contaminants Freshly Amended or Weathered and Aged in Sassafras Sandy Loam Soil Determined Using the Same Testing Protocols for Enchytraeid Reproduction Test with *Enchytraeus crypticus*.

Exposure/Benchmark	CL-20[a]	TNB[b]	2,4-DNT[b]	2,6-DNT[b]	TNT[c]	RDX[d]	HMX[d]
Freshly amended							
EC_{50}	0.3	11	36	57	98	51,413	>21,750[e]
EC_{20}	0.1	5	19	37	77	3,715	>21,750[e]
Weathered and aged							
EC_{50}	0.1	22	27	29	48	142,356	>17,498[e]
EC_{20}	0.035	9	14	18	38	8,797	>17,498[e]

Table notes: [a] Data from this study; [b] Kuperman et al., 2004; [c] Kuperman et al., 2005; [d] Kuperman et al., 2003, [e] The highest concentration tested.

The effects of weathering and aging of contaminant explosives in soil on the exposure of terrestrial organisms and resulting soil toxicity have not been sufficiently investigated. No effect of weathering and aging of nitramines RDX or HMX in SSL soil on toxicity were reported for *E. crypticus* (Kuperman et al., 2003) and *Eisenia fetida* (Simini et al., 2003). Kuperman et al. (2004, 2005) reported that weathering and aging of nitroaromatic explosives and related compounds in SSL soil significantly increased the toxicity of TNT or 2,6-dinitrotoluene (2,6-DNT) to *E. crypticus*, while toxicities of 2,4-dinitrotoluene (2,4-DNT) or 1,3,5-trinitrobenzene (TNB) were unaffected. In contrast, Dodard et al. (2003) found decreased TNT toxicity to *E. albidus* in the Organization for Economic Cooperation and Development (OECD) artificial soil following a 21-d aging period. Decreased toxicity to collembolan *Folsomia candida* was reported for TNT aged in LUFA 2.2 soil at 60% of the WHC and 20°C in the dark (Schäfer, 2002). Direct comparison of those results to findings of our studies is difficult due to several factors, including differences in molecular structures, toxicities, and fate in soil of these energetic contaminants compared to CL-20, other authors' use of different test species, different soil types, and shorter or undefined aging periods.

Specific mechanisms for changes in the toxicity following weathering and aging of CL-20 in soil are not well understood. Compounds produced due to CL-20 degradation or transformation during the weathering and aging process may be more toxic or more bioavailable to soil organisms compared with the parent material, and these may be factors contributing to the increased toxicity in weathered and aged treatments. Increased toxicity of metabolic byproducts of energetic soil contaminants was demonstrated by Lachance et al. (2004) in a study investigating the effects on adult *Eisenia andrei* of TNT and its reduction products 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), 2,4-diamino-6-nitrotoluene (2,4-DANT) and 2,6-diamino-4-nitrotoluene (2,6-DANT). The authors reported LC_{50} values for adult mortality from exposure to TNT, 4-ADNT, or 2-ADNT, of 132, 105, and 215 mg kg^{-1},

respectively, and gave the following order of toxicity: 4-ADNT > TNT > 2-ADNT. Our study does not allow determining whether increased toxicity of weathered and aged CL-20 soil treatments to *E. crypticus* was caused by formation of its degradation products in soil, as was demonstrated for TNT by Lachance et al. (2004), or alteration of bioavailability of the parent chemical. Information on degradation pathways of CL-20, and particularly on its fate in soil, is limited because investigations of this relatively new compound are ongoing (Bhushan et al., 2003, 2004a, b, c; Trott et al., 2003; Balakrishnan et al., 2004a, b; Hawari et al., 2004; Qasim et al., 2004; Szecsody et al., 2004).

Szecsody et al. (2004) reported that CL-20 abiotically degrades in oxic environments in the presence of 2:1 phyllosilicate clays (hectorite, montmorillonite, nontronite), micas (biotite, illite), and specific oxides (MnO_2, and the ferrous-ferric iron oxide magnetite). CL-20 was also found to be photoreactive when exposed to sunlight (Hawari et al., 2004; Szecsody et al., 2004). A few studies have shown that CL-20 can be biodegraded in aerobic surface soils (Jenkins et al., 2003; Trott et al., 2003) by bacteria isolated from these soils (Bhushan et al., 2003; Trott et al., 2003), and by functionally diverse bacterial enzymes (Bhushan et al., 2004a, b). Crocker et al. (2005) has shown that CL-20 is susceptible to both biotic and abiotic degradation in aerobic soils, with relatively rapid rates of CL-20 biodegradation (half-lives = 0.6 to 31.5 d) in surface and subsurface soils. Similar rates of CL-20 biodegradation were observed with garden and agricultural soils (Trott et al., 2003), however considerably slower rates of CL-20 degradation (half-lives = 144 to 686 d) were observed by Jenkins et al. (2003) with three firing range soils. The end products of CL-20 degradation in non-sterile or attenuated soils have commonly included nitrite (NO_2^-), nitrate (NO_3^-), nitrous oxide (N_2O), nitrogen gas (N_2), ammonia (NH_3), formate (HCOOH), and glyoxal (HCOCHO), although formation rates of the actual products depended on the specific abiotic or biotic processes (Balakrishnan et al., 2003, 2004b; Bhushan et al., 2003, 2004a, c; Trott et al., 2003; Hawari et al., 2004; Monteil-Rivera et al., 2004; Szecsody et al., 2004; Crocker et al., 2005). Several of these products, including glyoxal, ammonia, and formate, as well as the early reactive intermediates such as free radicals and imines (Balakrishnan et al., 2004a; Bhushan et al., 2004b, c; Hawari et al., 2004) can be formed during weathering and aging of CL-20 in SSL. These products of CL-20 degradation can be more toxic to enchytraeids compared with parent compound, thus potentially contributing to greater toxicity observed in our study with weathered and aged CL-20 treatments. Resolving CL-20 degradation pathways that lead to formation of toxic products, their fate in aerobic soils, and assessment of the individual toxicities of degradation products to soil receptors require further investigations to better understand the mechanisms of CL-20 toxicity in the soil vadose zone.

4. CONCLUSIONS

Results of our studies showed that toxicity of CL-20 to *Enchytraeus crypticus* in a natural soil, Sassafras sandy loam, was orders of magnitude greater compared with the currently used explosives RDX, HMX, and TNT. This soil type has low organic matter and clay contents, thus supporting relatively high contaminant bioavailability, necessary for developing conservative toxicity benchmark data that will be protective of ecological receptors in soil. Our findings of increased toxicity to *E. crypticus* of soil containing CL-20 weathered and aged in soil

clearly show that additional studies are required to investigate the toxicity of the CL-20 degradation products. Analogously, further investigation of the more toxic degradation compounds that arise within soils amended with CL-20 should also have a weathering and aging component, so that the level of persistence and long-term impact of the ecotoxicity of these degradation products may also be assessed.

All ecotoxicological parameters for *E. crypticus* were established on the basis of analytically determined concentrations of CL-20 in soil. Consequently, toxicological benchmarks determined using reproduction measurement endpoints will be used for establishing benchmark values for the derivation of draft Ecological Soil Screening Levels for soil invertebrates, and for use in Ecological Risk Assessment should specific terrestrial habitats become contaminated with CL-20. Overall results of this investigation strongly indicate that accidental release of CL-20 into the environment and concomitant ecosystem exposure can have detrimental effects on resident ecological receptors. This information should be considered by the manufacturer, potential users, risk assessors, and future site managers, during proposed periods of transition to CL-20 for military products that currently use energetic cyclic nitramines RDX and HMX, or the nitroaromatic explosive TNT.

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